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DOI:
10.1128/JVI.01552-09

JOURNAL OF VIROLOGY, Dec. 2009, p. 12407–12414 Vol. 83, No. 23
0022-538X/09/$12.00 doi:10.1128/JVI.01552-09

Hepatoma Cell Density Promotes Claudin-1 and Scavenger Receptor BI Expression and Hepatitis C Virus Internalization▽

Anne K. Schwarz, Joe Grove, Ke Hu, Christopher J. Mee, Peter Balfe,* and Jane A. McKeating

Hepatitis C Research Group, Institute For Biomedical Research, University of Birmingham, Birmingham B15 2TT, United Kingdom

Received 27 July 2009/Accepted 15 September 2009

Hepatitis C virus (HCV) entry occurs via a pH- and clathrin-dependent endocytic pathway and requires a number of cellular factors, including CD81, the tight-junction proteins claudin 1 (CLDN1) and occludin, and scavenger receptor class B member I (SR-BI). HCV tropism is restricted to the liver, where hepatocytes are tightly packed. Here, we demonstrate that SR-BI and CLDN1 expression is modulated in confluent human hepatoma cells, with both receptors being enriched at cell-cell junctions. Cellular contact increased HCV pseudoparticle (HCVpp) and HCV particle (HCVcc) infection and accelerated the internalization of cell-bound HCVcc, suggesting that the cell contact modulation of receptor levels may facilitate the assembly of receptor complexes required for virus internalization. CLDN1 overexpression in subconfluent cells was unable to recapitulate this effect, whereas increased SR-BI expression enhanced HCVpp entry and HCVcc internalization, demonstrating a rate-limiting role for SR-BI in HCV internalization.

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus, classified in the genus *Hepacivirus* of the family *Flaviviridae*. Worldwide, approximately 170 million individuals are persistently infected with HCV, and the majority are at risk of developing chronic liver disease. Hepatocytes in the liver are thought to be the principal reservoir of HCV replication. HCV pseudoparticles (HCVpp) demonstrate a restricted tropism for hepatocyte-derived cells, suggesting that virus-encoded glycoprotein-receptor interactions play an important role in defining HCV tissue specificity.

Recent evidence suggests that a number of host cell molecules are important for HCV entry: the tetraspanin CD81; scavenger receptor class B member I (SR-BI) (reviewed in reference 11); members of the tight-junction protein family claudin 1 (CLDN1), CLDN6, and CLDN9 (12, 34, 48, 52); and occludin (OCLN) (2, 33, 40). HCV enters cells via a pH- and clathrin-dependent endocytic pathway; however, the exact role(s) played by each of the host cell molecules in this process is unclear (4, 8, 21, 34, 45).

CD81 and SR-BI interact with HCV-encoded E1E2 glycoproteins, suggesting a role in mediating virus attachment to the cell (reviewed in reference 44). In contrast, there is minimal evidence to support direct interaction of CLDN1 or OCLN with HCV particles (12). Evans and colleagues proposed that CLDN1 acts at a late stage in the entry process and facilitates fusion between the virus and host cell membranes (12). We (13, 19) and others (9, 48) have reported that CLDN1 associates with CD81, suggesting a role for CLDN1-CD81 complexes in viral entry. Cukierman et al. recently reported that CLDN1 enrichment at cell-cell contacts may generate specialized membrane domains that promote HCV internalization (9). In this study, we demonstrate that cellular contact modulates SR-BI and CLDN1 expression levels and promotes HCV internalization. CLDN1 overexpression in subconfluent cells was unable to recapitulate this effect, whereas increased SR-BI expression enhanced HCVpp entry and HCVcc internalization rates, demonstrating a critical and rate-limiting role for SR-BI in HCV internalization.

MATERIALS AND METHODS

Cells and reagents. 293T cells (obtained from the ATCC) and Huh-7.5 cells (provided by C. Rice, Rockefeller University) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. The primary antibodies used were anti-NS5A 9E10 (C. Rice, Rockefeller University), anti-E2 (C1; D. Burton, Scripps Institute), anti-CD81 (M38; F. Berditchevski, University of Birmingham, United Kingdom), anti-SR-BI (ClaI; BD Transduction Laboratories), rabbit anti-SR-BI sera (T. Huby, INSERM, Paris), anti-CLDN1 JAY.8 (Invitrogen), anti-OCLN (Invitrogen), and anti-zonula occludens protein 1 (ZO-1) (Invitrogen). Fluorescence-labeled secondary antibodies, Alexa Fluor 488 anti-mouse and anti-rabbit 488 immunoglobulin G (IgG), were obtained from Invitrogen; horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare, United Kingdom.

Laser scanning confocal microscopy. Huh-7.5 cells were plated on collagen-treated 13-mm-diameter borosilicate coverslips (Fisher Scientific, United Kingdom) and fixed in methanol (CLDN1 and SR-BI) or 3% paraformaldehyde (CD81) 24 h postseeding. Primary antibodies were applied for 1 h or overnight (SR-BI) at 4°C. After the coverslips were washed twice with phosphate-buffered saline (PBS), anti-mouse Alexa Fluor 488 (Invitrogen, CA) secondary antibody was applied for 1 h. Following three washes with PBS, the cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) for visualization of nuclei, and the coverslips were mounted on glass slides (ProLong Gold antifade; Invitrogen). The cells were viewed by laser scanning confocal microscopy on a Zeiss Meta head confocal microscope with a 63× water immersion objective.

Linear-plot profile analysis. Receptor expression was monitored and quantified using linear-plot profile analysis in ImageJ (v1.83). Briefly, a horizontal line of 1-pixel thickness was drawn across the cell of interest, ensuring it crossed the plasma membrane on both sides. A list of *x*/*y* (distance/brightness) values was used to generate a linear-plot profile histogram using the microscope's built-in software. Five values were recorded per peak (representing the plasma membrane), and 10 random values were recorded for the area between the peaks (representing the cytoplasm). Fifteen to 20 images were acquired for each re-

* Corresponding author. Mailing address: Hepatitis C Research Group, Institute for Biomedical Research, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: (44) 121 414 8174. Fax: (44) 121 414 3599. E-mail: p.balfe@bham.ac.uk.

▽ Published ahead of print on 23 September 2009.

ceptor at each cell density. Brightness data were expressed as arbitrary fluorescence units (AFU), as previously described (19).

HCVpp generation and infection. Pseudoviruses were generated by transfecting 293T cells with plasmids carrying a human immunodeficiency virus provirus expressing luciferase or green fluorescent protein (GFP) and the HCV strain H77 or JFH E1E2 region, the murine leukemia virus (MLV) envelope, or a no-envelope control as previously described (21). The supernatants were harvested at 48 h posttransfection, clarified, and filtered through a 0.45-μm membrane. Virus-containing medium was added to Huh-7.5 cells plated in 48-well plates at 7.5×10^3, 15×10^3, or 30×10^3 cells/cm^2 and incubated for up to 8 h; thereafter, unbound virus was removed, and the medium was replaced with DMEM-3% FBS. At 72 h postinfection, the cells were assessed for GFP expression by flow cytometry or lysed with cell lysis buffer (Promega, Madison, WI), and luciferase activity measurements were taken for 10 s in a luminometer (Lumat LB 9507). Specific infectivity was calculated by subtracting the mean no-envelope signal from HCVpp or MLVpp signals.

HCVcc generation and infection. Plasmids encoding full-length JFH-1 (provided by T. Wakita, National Institute of Infectious Diseases, Japan) and J6/JFH (provided by C. Rice, Rockefeller University, NY) (25, 32, 47) were used to generate RNA as previously described (32). Briefly, RNA was transcribed using the RiboMax express T7 kit (Promega) and electroporated into Huh-7.5 cells. The supernatants were collected at 72 and 96 h postinfection, pooled, and stored at −80°C. High-titer viral stocks were generated by three serial passages through Huh-7.5 cells. The cells were fixed with methanol, and infected cells were identified by staining them for NS5A with the anti-NS5A monoclonal antibody (MAb) 9E10 and an Alexa 488-conjugated anti-mouse IgG. Infection was quantified by enumerating NS5A-positive foci, and infectivity was defined as the number of focus-forming units (FFU)/ml.

Neutralizing-antibody-dependent HCVcc internalization assay. Huh-7.5 cells were seeded in 48-well plates at 7.5×10^3, 15×10^3, or 30×10^3 cells/cm^2. Twenty-four hours postseeding, the cells were incubated on ice with JFH-1 or J6/JFH diluted in DMEM-3% FBS. After 1 h, unbound virus was removed by washing the plates thoroughly with ice-cold PBS. and entry was initiated by shifting the cells to 37°C. Internalization of cell-bound particles was blocked by adding neutralizing antibodies (nAbs) at different time point(s) after entry initiation. Infection was quantified by enumerating NS5A$^+$ foci at 48 h postinfection. The percent HCVcc internalization was calculated relative to the infectivity measured when nAbs were added 2 h post-temperature shift.

Proteinase K-dependent HCVcc internalization assay. Huh-7.5 cells (2×10^5) were pelleted at 1,200 rpm for 3 min and infected with JFH-1 or J6/JFH diluted in ice-cold HEPES-buffered DMEM-3% FBS. The cells were incubated for 1 h on ice; thereafter, unbound virus was removed by washing the cells thoroughly with ice-cold PBS, and the cells were resuspended in ice-cold HEPES-buffered DMEM-3% FBS. Entry was initiated by elevating the temperature to 37°C. Internalization of cell-bound particles was blocked by incubating the cells in ice-cold HEPES-buffered DMEM-3% FBS containing 50 μg/ml proteinase K (Sigma-Aldrich) on ice for 1 h. Posttreatment, the cells were washed twice with ice-cold HEPES-buffered DMEM-10% FBS to inactivate proteinase activity, counted, and reseeded at 15×10^3 cells/cm^2 in 24-well tissue culture plates. Infection was quantified by enumerating NS5A$^+$ foci at 48 h postinfection. The percent HCVcc internalization was calculated relative to the infectivity measured for untreated cells at each time point.

Generation of Huh-7.5 cells overexpressing SR-BI and CLDN1. Fully sequenced human SR-BI and CLDN1 genes were cloned into pTRIP lentiviral packaging plasmids as previously described (50). TRIP lentiviruses expressing SR-BI or CLDN1 were generated by cotransfecting 293T cells with plasmids encoding vesicular stomatitis virus G protein, human immunodeficiency virus Gag-Pol, and the pTRIP construct (1:3:3 ratio). Huh-7.5 cells were seeded at 1.5×10^5 cells per well in a six-well plate and infected 24 h later with the packaged lentivirus diluted in DMEM supplemented with 3% FBS for 12 h. The transduced cells were propagated in DMEM-10% FBS, and receptor expression levels were monitored by flow cytometry.

Western blotting. Huh-7.5 cells were plated in 10-cm-diameter plastic culture dishes at 7.5×10^3 or 30×10^3 cells/cm^2 and, 24 h postseeding, lysed in RIPA buffer containing protease (Complete; Roche) and phosphatase (PhoStop; Roche) inhibitors for 30 min on ice. Samples were cleared by centrifugation (20,000 × *g*; 15 min), and the protein concentration was determined using BCA Protein Assay Reagent (Pierce) according to the manufacturer's instructions. Normalized protein quantities were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes for incubation with anti-CD81, anti-SR-BI, anti-CLDN1, or anti-β-actin (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence according to the manufacturer's instructions (Geneflow).

RESULTS

Cellular contact modulates HCV receptor expression and localization. To assess the expression and localization of HCV receptors, Huh-7.5 cells were seeded at subconfluence (7.5×10^3 cells/cm^2; cellular contact between <20% of neighboring cells) or confluence (30×10^3 cells/cm^2; intact monolayer) on glass coverslips with CD81, CLDN1, or SR-BI or imaged by confocal microscopy (Fig. 1A). CLDN1 and SR-BI predominantly localized to cellular junctions and showed minimal staining of single cells at the plasma membrane, whereas CD81 localization to the plasma membrane was independent of cellular contact. Quantitative analysis of receptor expression by pseudolinear profiling of individual cells confirmed that CLDN1 and SR-BI cell contact expression was significantly enhanced in confluent compared to subconfluent cells (Fig. 1B) (unpaired *t* test; $P < 0.001$), while CD81 expression was unaltered (Fig. 1C). No discernible increase in cytoplasmic CLDN1 or SR-BI levels was observed, suggesting that cellular contact specifically modulates CLDN1 and SR-BI expression at the cell surface. Western blotting analysis of cell lysates from subconfluent or confluent cells demonstrated increased levels of CLDN1 and SR-BI, but not CD81, at cell confluence (Fig. 1D). Cell surface expression of the tight-junction proteins OCLN and ZO-1 was unchanged with cell confluence (Fig. 1E). CLDN1 and SR-BI mRNA levels in subconfluent and confluent cells were unchanged (data not shown), suggesting that cell density-specific regulation of CLDN1 and SR-BI expression occurs in a posttranscriptional manner.

To determine the effect(s) of increased SR-BI and CLDN1 cell contact expression on HCV infection, subconfluent and confluent Huh-7.5 cells were inoculated with JFH-1 or J6/JFH HCVcc for up to 6 h, and infectivity was determined 48 h postinfection (Fig. 2A). It should be noted that under all assay conditions the multiplicity of infection was approximately 0.3; therefore, the number of target cells did not limit infection (24, 36). JFH-1 and J6/JFH infectivity increased linearly over time, with both viral strains showing infection rates of ~5,700 FFU/ml/h at confluence compared to ~2,700 FFU/ml/h at subconfluence. Consistent with increased CLDN1 and SR-BI levels, we measured a twofold increase in the number of JFH-1- and J6/JFH-infected foci following a 6-h inoculation period in confluent compared to subconfluent cells, suggesting a relationship between target cell density, i.e., the establishment of cellular contact, and HCV infectivity. Like that of HCVcc, HCVpp-JFH (genotype 2a) and HCVpp-H77 (genotype 1a) infectivity increased with prolonged inoculation time (Fig. 2B), suggesting that attachment and/or internalization may be rate limiting for viral entry. To ascertain whether this was mediated at the level of viral entry, we measured the ability of HCVpp to infect target cells seeded at subconfluence at standard seeding density (15×10^3 cells/cm^2; ~40% cell-cell contact) or at confluence. A modest increase in HCVpp luciferase activity was observed between the subconfluent and standard seeding densities, with a further twofold increase when 100% cell contact was established (Fig. 2C). The infectivity of MLVpp also increased significantly from subconfluent to standard

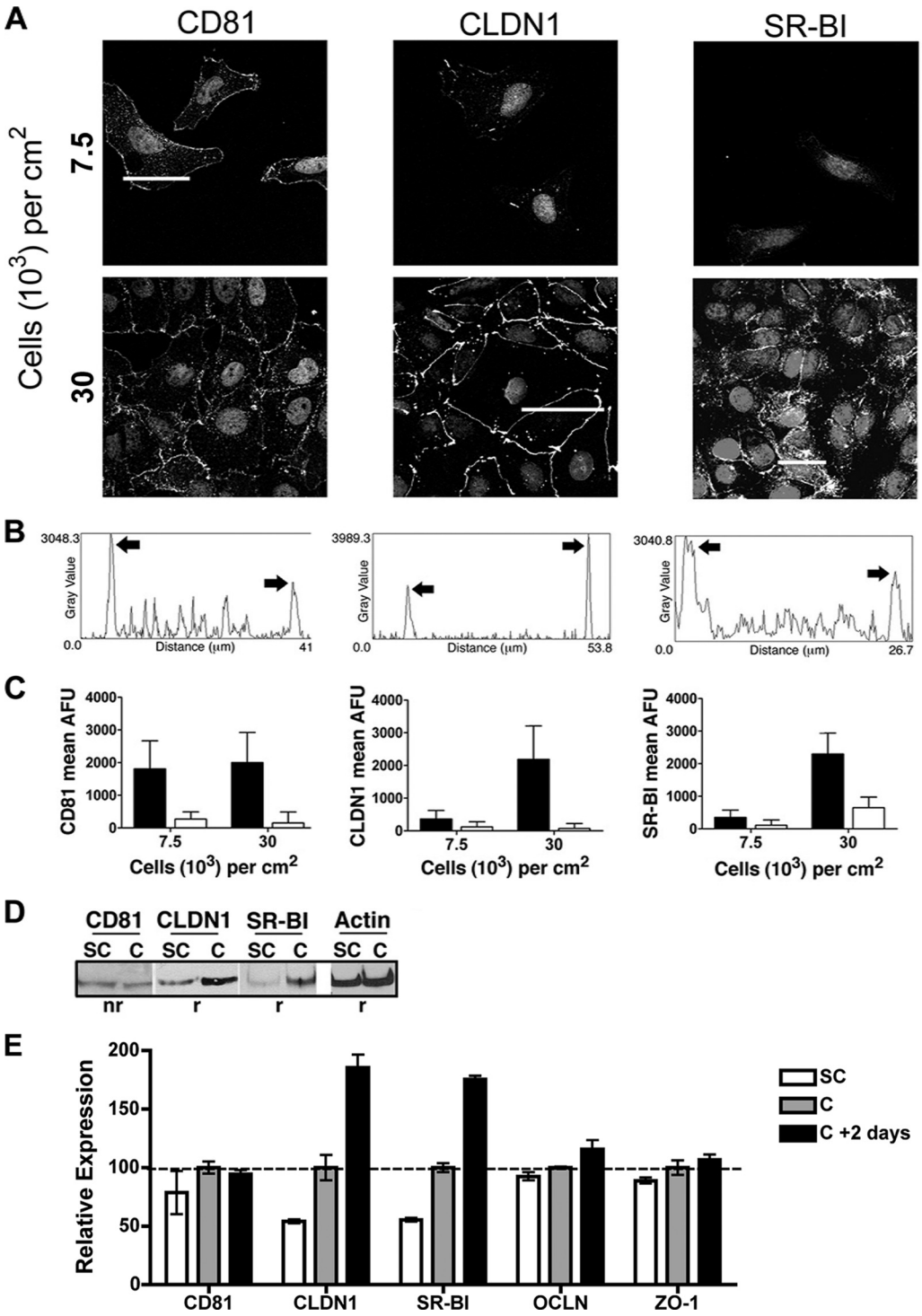

FIG. 1. Receptor expression is modulated in confluent Huh-7.5 cells. (A) Subconfluent and confluent cells were stained with antibodies specific for CD81, CLDN1, and SR-BI. The images were taken at ×63 magnification with a 1.3-numerical-aperture objective. Representative linear-profile plots are indicated by the white lines. (B) Linear-profile histograms; the arrows indicate plasma membrane staining. (C) Receptor expression at the plasma membrane (black bars) and cytoplasm (white bars). A minimum of 15 cells were analyzed per receptor at each seeding density. The error bars indicate standard deviations. (D) Western blot analysis of protein expression in subconfluent (SC) and confluent (C) Huh-7.5 cells. Five micrograms of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing (nr) or reducing (r) conditions, transferred to membranes, and probed for CD81, CLDN1, SR-BI, or actin. (E) Huh-7.5 cells were seeded at 4×10^3 cells/cm^2, and HCV receptor protein(s) (CD81, CLDN1, SR-BI, or OCLN) and tight-junction protein ZO-1 expression was quantified at subconfluence (SC), confluence (C), and confluence plus 2 days. Receptor expression at the various time points is plotted relative to the mean number of AFU for confluent cells. A minimum of 20 cells were analyzed for each protein at each time point.

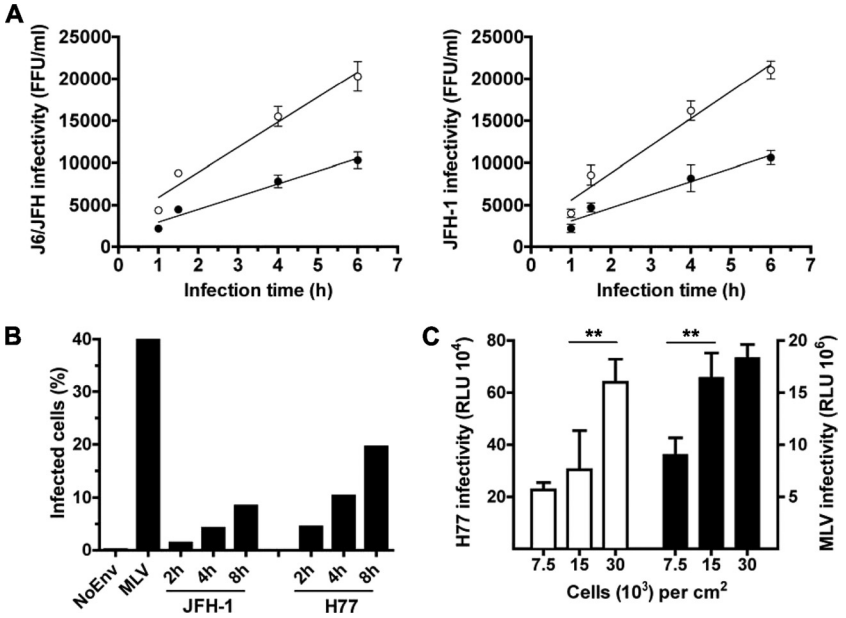

FIG. 2. Effect of cellular contact on HCVcc and HCVpp infection. (A) Huh-7.5 cells were plated at subconfluence (●) (7.5×10^3/cm^2) or confluence (○) (30×10^3/cm^2) and 26 h postplating were infected with JFH-1 or J6/JFH HCVcc at an approximate multiplicity of infection of 0.3 for 1 to 6 h. Forty-eight hours postinfection, infectivity was quantified by counting NS5A-positive foci. The values represent means ± standard deviations. (B) Huh-7.5 cells at standard seeding density (15×10^3 cells/cm^2) were infected with HCVpp-JFH or HCVpp-H77, envelope-deficient pseudoparticles (NoEnv), and MLVpp for up to 8 h at an approximate multiplicity of infection of 0.1. Viral infectivity (percent GFP-positive cells) was determined by flow cytometry. (C) Huh-7.5 cells were plated at different densities and infected with HCVpp-H77 or MLVpp for 5 h. Luciferase activity (in relative light units [RLU]) was measured 72 h postinfection. Statistical analyses were performed using unpaired *t* tests. **, $P < 0.01$.

seeding densities; however, only a modest further increase in luciferase activity was observed when cellular contact was established.

HCV internalization kinetics in confluent cells. To ascertain whether cellular contact promotes HCV internalization, we studied the kinetics of viral escape from the neutralizing effect of the anti-E2 MAb C1. HCVcc was pre-bound to Huh-7.5 cells on ice to prevent internalization. To initiate entry, the cells were then shifted to 37°C, and C1 MAb was added at different time points thereafter. The number of infected foci reached a plateau at 2 h after entry initiation, indicating that 100% of the bound virus was resistant to neutralization (Fig. 3A). JFH-1 and J6/JFH escape rates from neutralizing C1 MAb were comparable, with 23-min and 18-min half-maximal internalization rates ($t_{50\%}$) at standard seeding density, respectively (Fig. 3B). JFH-1 demonstrated comparable escape from a pooled preparation of IgGs purified from HCV-infected subjects and to C1 MAb, with similar $t_{50\%}$ (17 min and 22 min, respectively) (Fig. 3C), confirming that the observed escape kinetics were not specific for the C1-mediated inhibition of E2-receptor interaction(s). Similar JFH-1 $t_{50\%}$ (12 min) were measured using a proteolytic escape assay, which monitors the resistance of internalized virus to proteinase K (Fig. 3D). Unfortunately, the amount of proteinase K required to remove infectious HCVcc from Huh-7.5 hepatoma cells induced their dissociation from the tissue culture surface, requiring the cells to be replated during the experiment. To study the effect of cellular contact on HCV internalization kinetics, we compared JFH-1 infection of subconfluent and confluent Huh-7.5 cells and escape from C1 (Fig. 4). At subconfluence, 50% of cell-bound particles became resistant to C1 MAb neutralization within 30 min after entry initiation. However, when cellular contact was established, the half-maximal entry time of JFH-1 was reduced to 15 min, representing a significantly accelerated internalization rate ($P < 0.031$; paired *t* test).

Roles of CLDN1 and SR-BI in HCV internalization. To assess the significance of CLDN1 and SR-BI overexpression for HCV internalization, Huh-7.5 cells were transduced with lentiviral vectors expressing human SR-BI or CLDN1, and virus internalization was determined by means of the nAb escape assay. Transduced cells demonstrated a significant increase in CLDN1 and SR-BI expression at cell-cell junctions compared to parental cells (Fig. 5). In addition, a relatively large proportion of receptor molecules were retained intracellularly in both transduced cell populations (Fig. 5A). We noted that overexpression of both CLDN1 and SR-BI negated the effects of cell confluence on receptor expression. There was no detectable effect(s) of CLDN1 overexpression on SR-BI or CD81 levels, and vice versa (data not shown). Attempts to generate a Huh-7.5 cell line overexpressing CD81 failed, most likely because human hepatoma cells already express relatively high levels of CD81.

At standard seeding density, overexpression of SR-BI increased Huh-7.5 susceptibility to JFH-1 HCVcc infection threefold (Fig. 5C), consistent with previous findings (18), whereas increased CLDN1 expression had no detectable effect. This suggests that SR-BI, but not CLDN1, expression levels limit the permissivity of Huh-7.5 cells to JFH-1 HCVcc. To assess whether high levels of CLDN1 or SR-BI altered the internalization kinetics of cell-bound virus, we measured C1 escape in transduced Huh-7.5 cells plated at standard seeding density (Fig. 6). CLDN1 overexpression had no effect on

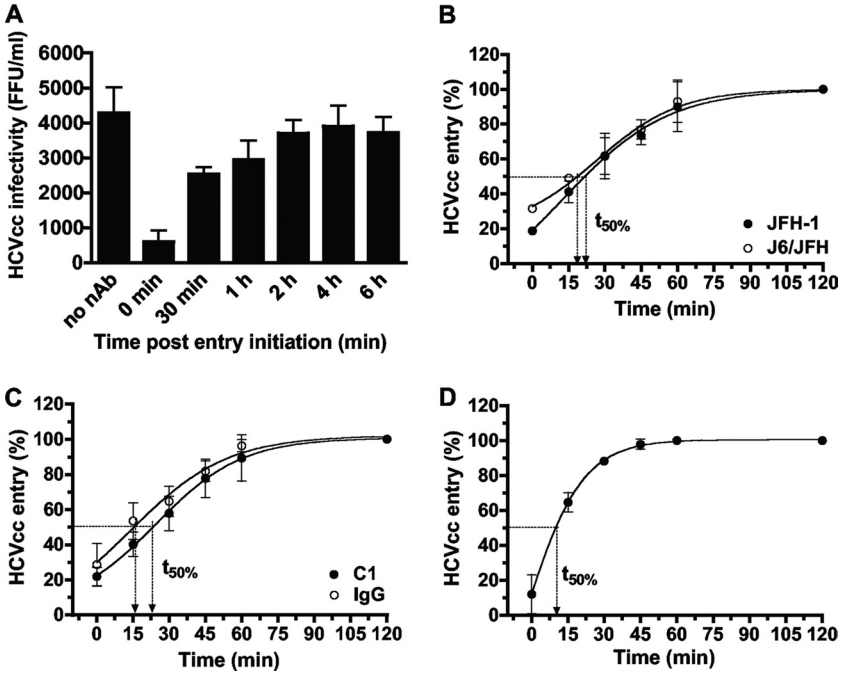

FIG. 3. Time course of HCVcc sensitivity to antibody-mediated neutralization. (A) Neutralizing activity of the anti-E2 nAb C1. Huh-7.5 cells plated at standard seeding density (15×10^3 cells/cm^2) were incubated with JFH-1 at an approximate multiplicity of infection of 0.3 for 1 h on ice; thereafter, entry was initiated by elevating the temperature to 37°C. At different time points after entry initiation, virus infectivity was neutralized using 10 μg/ml C1. Infectivity (FFU) reached a plateau approximately 2 h after entry initiation. The data are from a representative experiment performed in quadruplicate. The error bars indicate standard deviations. (B) JFH-1 and J6/JFH internalization. Huh-7.5 cells plated at standard seeding density (15×10^3 cells/cm^2) were infected with JFH-1 or J6/JFH for 1 h on ice, and entry was initiated as described for panel A. To neutralize further infection, an anti-E2 antibody (C1) was added to the cells at the indicated time points. (C) Neutralization with anti-E2 antibody and polyclonal anti-HCV human IgG. Huh-7.5 cells were infected with JFH-1 as described for panel B. Virus infectivity was neutralized using anti-E2 antibody (C1) or a mixture of polyclonal human IgGs. Infectivity was enumerated 48 h later. The arrows show the $t_{50\%}$. (D) Neutralization with proteinase K. Huh-7.5 cells were infected with JFH-1 as described for panel B, and cell surface-bound virus was inactivated by proteinase K digestion (50 μg/ml) at the indicated time points. The values are relative to the infectivity measured at 2 h after entry initiation (the values shown are the means of three experiments, each with observations in triplicate; the error bars indicate standard deviations).

JFH-1 internalization, whereas in SR-BI-overexpressing cells, 50% of cell-bound particles became resistant to neutralization within 6 min after entry initiation compared to a $t_{50\%}$ of 18 min for parental cells. These data highlight the crucial role of SR-BI for HCV entry and suggest that receptor levels define virus internalization rates.

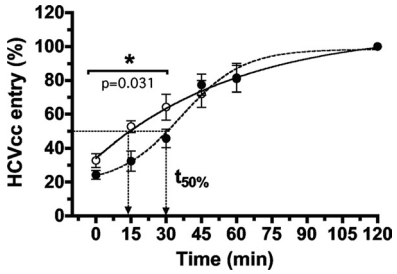

FIG. 4. Modulation of HCVcc entry kinetics with cell confluence. Huh-7.5 cells were plated at subconfluence (●) (7.5×10^3 cells/cm^2) or confluence (○) (30×10^3 cells/cm^2) and incubated with JFH-1 at an approximate multiplicity of infection of 0.3 for 1 h on ice. After entry initiation, internalization was blocked with anti-E2 antibody (C1) at the indicated time points. Infectivity was determined 48 h postinfection. The values are relative to the infectivity measured at 2 h after entry initiation (the values shown are the means of three experiments, each with observations in triplicate; the error bars indicate standard deviations). Statistical analysis was performed using a two-tailed paired *t* test to compare percent entry at individual time points.

DISCUSSION

We demonstrated that HCV entry is dependent on the target cell density, and this is most likely explained by increased CLDN1 and SR-BI expression in areas of cell-cell contact. CLDN1 and SR-BI demonstrated significantly elevated cell contact localization and expression in confluent cells (Fig. 1), whereas mRNA levels were unaffected by the confluence status, suggesting posttranscriptional regulation of SR-BI and CLDN1 expression. In contrast, CD81 and OCLN demonstrated a uniform distribution at the plasma membrane of single cells, with no detectable enrichment at high density (Fig. 1A and E). Western blot analysis of total cell lysates confirmed that CD81 protein levels were unaffected by the confluence status. However, Huh-7.5 cells express relatively high levels of CD81, and we presume that modest changes in CD81 expression may be undetectable. These findings suggest that the Huh-7.5 cell density modulates CLDN1 and SR-BI protein expression and localization with possible consequences for HCV entry.

Cell surface expression of membrane proteins involves the sorting of proteins from the *trans*-Golgi network to the plasma

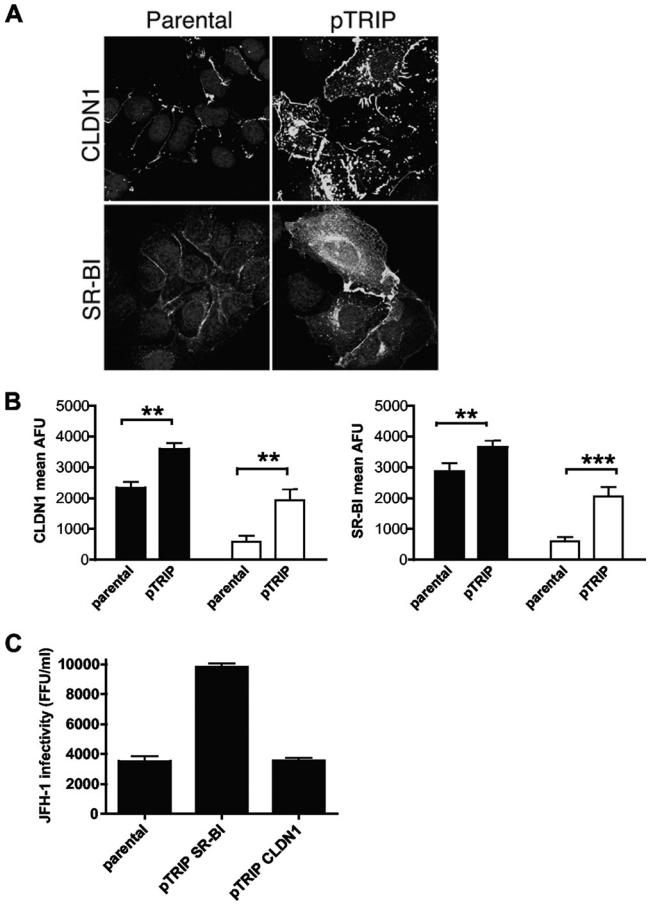

FIG. 5. CLDN1 and SR-BI overexpression in Huh-7.5 cells. (A) Parental and pTRIP-transduced cells were plated at standard seeding density (15×10^3 cells/cm^2) and, 26 h post-plating, were stained with antibodies specific for CLDN1 or SR-BI. (B) Receptor expression levels at the plasma membrane (black bars) and cytoplasm (white bars) were determined by linear-profile plot analysis. Statistical analysis was performed using an unpaired *t* test. The error bars indicate standard deviations. (C) Parental and transduced cells at standard seeding density were infected with JFH-1 at an approximate multiplicity of infection of 0.3 for 1 h. JFH-1 infectivity (FFU/ml) was determined by enumerating NS5A$^+$ foci at 48 h postinfection. Asterisks indicate statistical significance (**, $P < 0.01$; ***, $P < 0.001$).

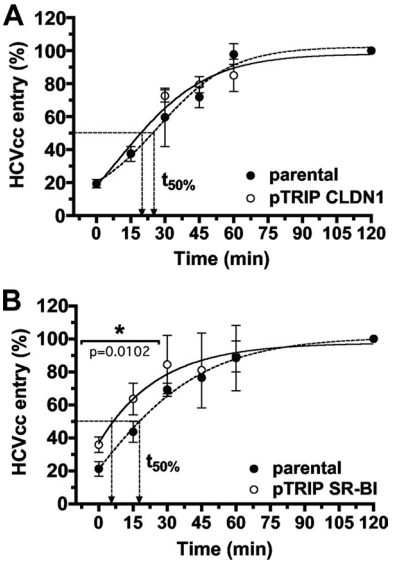

FIG. 6. Effect of CLDN1 and SR-BI overexpression on JFH-1 entry kinetics. Huh-7.5 cells transduced to overexpress CLDN1 (A) or SR-BI (B) were plated at standard seeding density and incubated with JFH-1 at an approximate multiplicity of infection of 0.3 for 1 h on ice. Entry was initiated by elevating the temperature to 37°C; thereafter, infection was further neutralized with anti-E2 antibody (C1). NS5A$^+$ foci were enumerated 48 h postinfection, and the percentage of C1-resistant particles was calculated relative to the infectivity measured at the 2-h time point. The values shown are the means of three experiments, each with observations in triplicate; the error bars indicate standard deviations.

membrane domain and anchoring and/or retention at the cell surface, followed by endocytosis, transcytosis, or recycling (reviewed in reference 5). Protein trafficking to the cell surface is largely mediated by peripheral membrane (PDZ domain) proteins, which interact with the C-terminal PDZ ligand binding domains of membrane proteins, including CLDN1 and SR-BI (23, 42). The mechanisms regulating membrane trafficking are incompletely understood; however, it has been demonstrated that adaptor proteins, such as PDZK1 and ZO-1 and -2, localize to sites of cell-cell contact and interact directly with CLDN1 and OCLN (14, 23, 26, 37).

There have been reports of CLDN1 cell contact localization in human, murine, and canine tumor and nontumor cell lines (16, 17, 20, 22, 29). Indeed, human breast cancer cells display cell density-specific modulation of CLDN1 expression independently of mRNA levels. Hepatic SR-BI expression is post-translationally regulated by the adaptor protein PDZK1 (27); furthermore, by stabilizing SR-BI plasma membrane expression, PDZK1 determines SR-BI localization and function within the liver (42, 43).

SR-BI and CD81 surface expression levels have been reported to limit HCV infection (18, 28). Consistent with these findings, HCVpp demonstrated a significant increase in luciferase activity at cell confluence, when cell-cell contact was established between cells in the culture (Fig. 2C). The same effect was observed for HCVcc infection, with JFH-1 and J6/JFH infection increasing significantly when cellular contact was established (Fig. 2). To assess whether cellular contact promoted the internalization of cell-bound HCVcc, we examined the ability of an anti-E2 antibody to inhibit infection of subconfluent and confluent cells when added at various times during the entry process. Previous reports have demonstrated that HCVpp and HCVcc escape the inhibitory activity of antibodies targeting HCV E2, CD81, and SR-BI approximately 17 to 20 min after entry initiation, consistent with the internalization rates reported in this study (3, 12, 49). Interestingly, Meertens and colleagues reported that half-maximal HCVpp fusion requires 73 min, as determined by sensitivity to bafilomycin A (34), suggesting a significant delay in virus internalization and subsequent fusion with early endosome membranes. We note that the inhibitory activity of C1 was reduced earlier at cell confluence than at subconfluence, with half-maximal inhibition being attained at 15 min and 30 min, respectively (Fig. 4). Moreover, we observed that HCVcc internalization in subconfluent and confluent cells differed significantly during the first 30 min of the entry process and

converged thereafter, suggesting that cellular contact modulates an early step in HCV internalization.

To further dissect the role(s) of CLDN1 and SR-BI in cell contact-mediated modulation of HCV entry, we studied HCVcc internalization in subconfluent Huh-7.5 cells transduced to overexpress CLDN1 or SR-BI. Cells transduced to overexpress CLDN1 were no more susceptible to JFH-1 HCVcc than parental cells (Fig. 5); furthermore, CLDN1 overexpression had no detectable effect on virus internalization (Fig. 6), despite a significant increase in cell contact expression levels. Taken together, these data suggest that CLDN1 expression levels do not limit the frequency or rate of HCVcc internalization into Huh-7.5 cells. CLDN1 is proposed to act at a late postbinding stage of viral entry (12). Several reports have demonstrated that mutation or deletion of a domain(s) required for CLDN1 internalization has minimal effect on HCV entry (9, 12, 19), suggesting that CLDN1 internalization per se is not essential for HCV particle entry. Our recent data suggest that CLDN1 may interact with CD81 and modulate HCV E1E2 interaction(s) and lateral particle trafficking to membrane domains amenable to internalization (H. Harris and M. Farquhar, unpublished data).

SR-BI overexpression enhanced HCV internalization (Fig. 5C), consistent with a model in which elevated SR-BI expression levels enhance HCV internalization in confluent cells. Silencing SR-BI expression reduced the infectivity of HCVcc (49) and HCVpp bearing E1E2 glycoproteins from different genotypes (30), while the neutralizing activity of patient sera increased as a result of low SR-BI levels (46). Concomitantly, we observed that SR-BI overexpression promoted HCVpp entry and "attenuated" the neutralizing activity of MAbs and polyclonal antibodies (reference 18 and data not shown). These observations suggest that SR-BI expression levels not only modulate Huh-7.5 permissivity per se (Fig. 5) (18), but are rate limiting for HCVcc particle internalization (Fig. 6). Compared to other enveloped viruses, HCVcc internalization is slow, which may reflect the time required to form higher-order protein complexes between CD81, SR-BI, CLDN1, and OCLN (10, 19, 34). High SR-BI surface expression may facilitate the assembly of these complexes, promoting the internalization of cell-bound particles. Of note, the anti-E2 MAb (C1) used to neutralize surface-bound virus particles (Fig. 3, 4, and 6) is reported to act by inhibiting E2-CD81 interactions (31) and yet retains its neutralizing capacity after particle attachment (Fig. 3A). This is consistent with other observations that CD81 acts at a stage following the primary interaction of HCV particles and target cells (12, 15, 35).

Persistence is a hallmark of HCV infection and is attributable to the ability of the virus to evade host cellular and humoral immune responses. The mechanism of action of HCV-specific nABs is incompletely understood; however, there have been several reports that antibodies inhibit HCV attachment to the cell surface by targeting the CD81 binding site and hypervariable region of glycoprotein E2 (1, 38, 39, 51). During viral entry, both regions need to be accessible to their cellular binding partners (6, 7) and may become transiently exposed to nAbs; consequently, the rate at which a virus particle internalizes may define its sensitivity to nAbs. Hepatocytes, which are the main reservoir of HCV replication, are tightly packed; furthermore, we have previously demonstrated high levels of SR-BI in healthy human liver (41). It is interesting to speculate that the internalization rates and nAb escape we have observed in confluent Huh-7.5 cells, with high SR-BI and CLDN1 expression, may better represent hepatocytes in the liver. If this is indeed the case, the rate of internalization of virus particles in vivo may be rapid, offering further explanation for the ability of HCV to prevail in the face of a humoral immune response.

ACKNOWLEDGMENTS

We thank T. Wakita for full-length and subgenomic JFH-1; C. Rice for J6/JFH, Huh-7.5 cells, and anti-NS5A 9E10; D. Burton for C1 anti-E2 MAb; and F. Berditchevski for anti-CD8.

This work was supported by the MRC, the Wellcome Trust, and PHS grants AI50798 and AI40034-14.

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